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Screening and Characterization of Biosurfactants Producing Microorganism formNatural Environment(Whey Spilled Soil)

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Abstract:
Biosurfactant are surface active agent produce by diverse group of microorganism. Disposal of whey is common in India especially in West Bengal, so in our study we collect sample form different whey spilled soil and isolate biosurfactant producing bacteria. Total ten different types of bacteria (APCCS1a-S5b) we have isolate for our study. Among them four are screened by growing them in CTAB agar plates and their hemolytic activity in blood agar plates. We analyses four strains for their ability to produce extracellular biosurfactant by oil spreading technique and finally confirmed by drop collapsing test. The extracellular biosurfactant of the isolated strains were extracted by conventional way and they are characterized by thin layer chromatography (TLC) and were shown that they are glycolipid ($APCCS_{1a}$, $APCCS_{3b}$ and $APCCS_{5a}$) and lipopeptide ($APCCS_{1b}$) in nature.The surface tension activities of extracted biosurfactant were evaluated by emulsification index (E_{24}). Finally we characterized the isolated strains according to Berge`s Manual and they are belonging to Pseudomonas ($APCCS_{1a}$ and $APCCS_{5a}$), Lactobacillus ($APCCS_{2b}$) and Bacillus ($APCCS_{1b}$) group.
Keywords: Biosurfactants, Thin layer chromatography (TLC), cetyltrimethylammonium bromide (CTAB), hemolysis, whey, emulsification index.

1. Introduction

Biosurfactants are surface active molecules having hydrophilic and hydrophobic moieties as their constituents which allow them to interact at interfaces and reduce the surface tension. They are produce by diverse group of organism belong to bacteria, fungi and actinomycetesetc., mainly on surfaces of microorganisms or may also secreted extracellularly. They are categorized based on their chemical composition as fatty acids, glycolipids, glycolipopeptides, glycoproteins, lipopeptides, phospholipids, polymeric and particulate biosurfactants. The chemicaldiversity of biosurfactants makes them a potential source for green chemicals having applications in industrial, environmental (agricultural and bioremediation), and medical fields. Almost all surfactants being currently produced are derived from petroleum source. However, these synthetic surfactants are usually toxic and hardly degraded by microorganisms. These are potential source of pollution and damage to the environment. Therefore, in the recent years, much interest and attention have been directed towards biosurfactants over chemically synthesized surfactants due to their superiority to the chemical surfactants with respect to their biocompatibility, lower toxicity, higher biodegradability, higher stability, extreme stability in extreme temperature and pH. With the advent of time, this attribute is contributing its higher demand in the field of biotechnology.

The agricultural waste such as whey (a by-product of the manufacture of cheese or casein) are well known for containing high levels of carbohydrates and of lipids -both of which are necessary for substrates for the production of biosurfactants and contains all necessary substances (lactose, protein, organic acids and vitamins) that require for growth of surfactant producing microorganism. This study focus on the screening, production, extraction and purification of biosurfactant from bacteria isolated from whey spilled soil and which is easily available in India.

2. Materials and methods

2.1. Sampling area

For isolation biosurfactant producing bacteria soil samples were collected from whey spilled surfaces of five different cheese making farm of West Bengal, India (sample 1-5). The samples were collected in sterile container under aseptic condition and were taken to the laboratory for analysis. The pH of the samples during collection was 7.0 and temperature was 30^0C.

2.2. Enrichment, Isolation and enumeration of bacterial isolates

5.0g of the whey spilled soil samples were dissolving in 100 ml of phosphate buffer saline (PBS). After precipitation of solid debris 5ml liquid suspension are inoculated in 50ml of nutrient broth and incubated at 25°C with agitation speed of 200 rpm for 48 hours.After incubation the medium was serially diluted from 10^{-1} to 10^{-6}

in sterile water. From the dilutions (10^{-1} to 10^{-6}) 1ml was transferred to sterile petri-dish containing 20mls of Reasoner´s 2A agar (R2A) contained (g/L): Proteose peptone, 0.5; Casamino acids, 0.5; Yeast extract, 0.5; Dextrose, 0.5; Soluble starch, 0.5; Dipotassium phosphate, 0.3; Magnesium sulfate $7H_2O$, 0.05; Sodium pyruvate, 0.3; Agar, 15; Final pH 7.2 ± 0.2 @ 25 °C, by spread plate techniques. The plates were then inverted and incubated at 25°C for 48 hours. Control and replica plates were maintained.

After incubation 30-300 colonies containing plates were selected and morphologically different colonies were streaked on LB agar media and obtained pure culture by incubating at 37^0C for 24 hours. The pure isolates were stored in R2A agar slants for further identification. These cultures were stored in R2A agar slants and kept under refrigerated condition (4^0C) for further experimentation.

2.3. Screening of biosurfactant producing organisms

The isolated colonies were tested for their biosurfactant production by different methods; CTAB Agar Plate;Oil Spreading Technique; Blood Hemolysis Test and Drop collapsing test.

2.3.1. CTAB Agar Plate

The CTAB agar plate method is a semi-quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner. Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg ml^{-1}) and methylene blue (5 mg ml^{-1}) were used to detect extracellular glycolipid production. Biosurfactants were observed by the formation of dark blue halos around the colonies.

2.3.2. Oil spreading technique

Isolate bacterial strains was incubated into 100 mL of culture medium. The Mckeen medium (20 gL^{-1} glucose, 5.0 gL^{-1} glutamic acid, 1.0 gL^{-1} K_2HPO_4, 1.02 gL^{-1} $MgSO_4$, 0.5 gL^{-1}KCl) supplemented with 1 mL of trace elements solution (0.5 gL^{-1} $MnSO_4$,.$7H_2O$, 0.16 gL^{-1} $CuSO_4$,.$5H_2O$ and 0.015 gL^{-1} $FeSO_4$,.$7H_2O$) adjusting to pH 7.0 was used as cultural medium. The cultures were incubated on rotary shaker (150 rpm) for 3 days at 25 °C. The culture suspension was screened for biosurfactant production by the oil spreading techniques (Anandaraj&Thivakaran, 2010; Priya&Usharani, 2009). The procedure is as follows: 30ml of distilled water was taken in the petri dish (25cm in diameter). 20µl of crude oil was added to the center of the plates containing distilled water. Now add 20µl of the supernatant of the culture suspension to the center. The biosurfactant producing organism can displace the oil and spread in the water. The diameter and area of clear halo visualized under visible light were measured and calculated after 1 minute.

2.3.3.Hemolytic activity test

Hemolytic activity appears to be a good screening criterion for surfactant-producing strains because biosurfactant producing capacity was found to be associated with hemolytic activity. The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. The plates were incubated for 48-72 hours at 37°C. Hemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996). These clear zones indicate the presence of bio-surfactants producing bacteria.

2.3.4.Drop collapsing test

Jain et al developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. The assay was done in following way: 2µl of mineral oil was added to each well of a 96-well micro-titer plate lid. The lid was equilibrated for 1 hour at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

2.4. Identification of bacteria

The isolated biosurfactant producing bacteria was characterizedby microbiological and biochemical tests such as Gram staining, carbohydrate fermentation test, H_2Sproduction test, indole production test, methylred test, Voges-Proskauer test, citrate utilization test, urease test,catalase test, oxidase test, litmus milk reaction, starch hydrolysistest, gelatin hydrolysis test, lipid hydrolysis test.

Results from the biochemical analysis were used to find the closest match with known bacterial genus and to assign the bacterial signature according to Bergey's manual.

2.5. Extraction of biosurfactants

Each culture was inoculated in 50 ml of Mckeenbroth with 1 ml of whey. The culture was incubated at 25^0C for 3 days with shakingcondition. After incubation the bacterialcells were removed by centrifugation at 8000rpm, 4^0C for 10 minutes. Thesupernatant was taken and the pH of thesupernatant was adjusted to 2, using 1MH_2SO_4.

Now add equal volume ofchloroform: methanol (2:1). This mixturewas shaken well for mixing and left overnight for evaporation. White colored sediment was obtained as a result i.e., the "Biosurfactants".

2.6. Recharacterization of biosurfactants

Preliminary characterization of the biosurfactant was done by Thin Layer chromatography (TLC) method. A spot of crude biosurfactant was placed on the silica plate(Merck & Co., Mumbai, India) and the biosurfactant was separated on the plate using chloroform: methanol: water (:10:0.5). The plate was developed with different color developing reagents. Ninhydrin reagent (0.5g ninhydrin in 100ml anhydrous acetone) was used to detect lipopeptide biosurfactant as red spots and anthrone reagent (1g anthrone reagent in 5ml sulfuric acid mixed with 95 ml ethanol) to detect glycolipid biosurfactant as yellow spots (Yin et al., 2008).

2.7. Emulsification index (E_{24})

The emulsifying capacity was evaluated by an emulsification index (E_{24}). The $E_{24}$of culturesamples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vortexing at high speed for 2 min and allowed to stand for 24h. The E index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm).The percentage of emulsification index calculated by using the following equation (Cooper and Goldenberg, 1987).

$$E = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \text{ X } 100$$

3. Result

In the spread from the serial dilution tubes the colonies were enumerated and listed in the Table: 1. Ten different types of colonies (2 from each samples) were identified morphologically and were purified and stored at 4^0C for further analysis.

3.1. CTAB Agar Plate

CTAB Agar Plates results showed the dark blue halos in the six isolates ($APCCS_{1a,\ 1b}$; $APCCS_{2b}$; $APCCS_{4a}$; $APCCS_{5a,\ 5b}$)indicating positive activity of Biosurfactant production (Table: 2).

3.2. Oil spreading technique

Cultures of ten isolated strains ($APCCS_{1a,\ 1b}$; $APCCS_{2a,\ 2b}$; $APCCS_{3a,\ 3b}$; $APCCS_{4a,\ 4b}$; $APCCS_{5a,\ 5b}$) were centrifuged and added to the oil containing plates. The strain $APCCS_{1a}$, $APCCS_{1b}$, $APCCS_{3b}$ and $APCCS_{5a}$ showed the clear zone by being able to displace the oil around the colony indicating biosurfactant production. No clear zone was observed with control. The results were tabulated (Table: 2).

3.3. Hemolytic Activity

The isolated were streaked in the blood agar plates. The hemolytic activity was observed in all the ten isolated strains, results showed (alpha) hemolytic activity of strain $APCCS_{2a}$, $APCCS_{3b}$,$APCCS_{4a}$,and $APCCS_{5b}$, the (beta) hemolytic activity of strain $APCCS_{1a}$, $APCCS_{1b}$, $APCCS_{2b}$and $APCCS_{5a}$andthe (gamma) hemolytic activity of strain$APCCS_{3a}$and$APCC_{4b}$(Table:2).

3.4. Drop collapsing test

The isolated culture supernatant are placed on an oil coated solid surface, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced indicate the presence of surfactant in the cell supernatants (Table:2).

3.5. Characterization of biosurfactant producing organisms

The screened biosurfactant producing organism was taken for the extraction surfactants. First the organism was identified by different biochemical tests. The results of it's were tabulated (Table: 3). Comparing the results with Bergey'sManual, identification of bacteria was performed.

3.6. Extraction of biosurfactants

The culture inoculated in Mckeen broth with whey was centrifuged and the supernatant was taken mixed with Chloroform: methanol. White sediment was retained while after evaporation for overnight.

3.7. Characterization of biosurfactants

The crude biosurfactant produced were characterized by using silica thin layer chromatography (TLC) plates. The sediment obtained was placed in the TLC plate and the plates when sprayed with ninhydrin reagent and anthrone reagent it showed red spot (for $APCCS_{1b}$) and yellow spots (for$APCCS_{1a}$,$APCCS_{2b}$ and $APCCS_{5a}$) in the plates respectively. This shows the production of lipopeptide (for $APCCS_{1b}$) and glycolipid (for$APCCS_{1a}$,$APCCS_{3b}$ and $APCCS_{5a}$)biosurfactants in the organisms.

3.8. Emulsification measurement

Emulsification activity was measured according to the method of Cooper and Goldenberg (1987). The emulsification activity of isolated strain was measure after 24 hours indicate the value varies from 45% to 60%. The results are given in table 4.

Table 1: ENUMERATION OF BACTERIAL STRAINS FROM THE SAMPLE

Dilution	Sample 1	Sample2	Sample 3	Sample 4	Sample5
10^{-1}	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-4}	282	192	137	227	209
10^{-5}	62	51	31	39	39
10^{-6}	TFTC	TFTC	TFTC	TFTC	TFTC
Average no of CFU/ml	$4.51x10^{6}$	$3.51\ x10^{6}$	$2.23\ x10^{6}$	$3.08\ x10^{6}$	$2.99\ x10^{6}$

Table 2: CHARACTERIZING BIOSURFACTANT PRODUCING BACTERIA

Isolated strains	**Growth in CTAB agar Plates**	**Haemolysis**	**Clear zone (mm) On Oil spreading technique**	**Drop collapsing test**
$APCCS_{1a}$	++	β- hemolytic	12	+
$APCCS_{1b}$	+	β- hemolytic	5	+
$APCCS_{2a}$	-	α- hemolytic	No zone	-
$APCCS_{2b}$	+	β- hemolytic	7	+
$APCCS_{3a}$	-	γ - hemolytic	No zone	-
$APCCS_{3b}$	-	α-hemolytic	No zone	-
$APCCS_{4a}$	+	α- hemolytic	No zone	-
$APCCS_{4b}$	-	γ - hemolytic	No zone	-
$APCCS_{5a}$	++	β- hemolytic	11	+
$APCCS_{5b}$	+	α-hemolytic	No zone	-
*S. mutans(+ve control for α-*hemolytic*)*	+	α-hemolytic	No zone	-
P.aeruginosa(+ve control for β-hemolytic)	++	β- hemolytic	10	+
E. coli (+ve control for γ-hemolytic)	-	γ- hemolytic	No zone	-

Table 3: IDENTIFICATION OF THE BACTERIAL CULTURE

Character		APCCS1a	APCCS1b	APCCS2b	APCCS5a
Morphology		Rod shaped	Rod shaped	Rod shaped	Rod shaped
Gram staining		-ve	+ve	+ve	-ve
Endospore staining		-ve	+ve	-ve	-ve
Motility test		+	+	-	+
carbohydrate fermentation test	Glucose	+	-	+	+
	Maltose	+	+	+	-
	lactose	-	-	+	-
	sucrose	-	-	+	+
	Fructose	-	-	+	-
	Mannitol	-	-	-	-
H_2S production test		-	-	+	+
indole production test		-	-	-	+
methyl red test		-	-	-	+
Voges-Proskauer test		-	+	-	-
citrate utilization test		+	+	-	+
urease test		-	+	-	+
catalase test		+	+	-	+
oxidase test		+	+	-	-
Nitrate reduction test		+	+	-	+
litmus milk reaction		+	+	+	+
starch hydrolysis test		-	+	-	-
gelatin hydrolysis test		-	+	+	+
lipid hydrolysis test		-	+	-	-
Strains Name (comparing with Berge`s Manual)		*Pseudomonas sp.*	*Bacillus sp.*	*Lactobacillus sp.*	*Pseudomonas sp.*

Table 4: **EMULSIFICATION MEASUREMENT**

Isolated strains	**Emulsification index (E_{24})**
$APCCS_{1a}$	63±0.4
$APCCS_{1b}$	33 ± 0.3
$APCCS_{2b}$	41±0.3
$APCCS_{5a}$	61±0.2
-veControl (*E. coli*)	00±0.2
+veControl (*P. aeruginosa*)	51±0.3

4. Discussion

Biosurfactant are potentially used recent years by different industry due to its nontoxic effects. The main aim of our study to isolate and characterizedbiosurfactant producing bacteria form whey spilled soil. Whey spillage soil contains all types of nutrient that require for growth of various types of microorganism along with bacteria those responsible for surfactant production. We have been able to isolate bacteria with the ability to produce biosurfactants from the soil samples collected from whey spillage area. The biosurfactants production ability of isolated bacteria are confirmed by different screening method including CTAB agar plate method, oil spreading technique, hemolytic activity and drop collapsing test.

Blue agar plate method is a semi quantitative agar plate method that is based on the formation of an insoluble ion pair of anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue. The isolated strains showed positive CTAB agar plate activity, indicate presence of extracellularbiosurfactant. The oil spreading technique was used as indicator for biosurfactant production as reported by Anandaraj&Thivakaran, 2010; Priya&Usharani, 2009 and successfully displace the oil was analyzed for isolated strains. The strains are further confirming for their surfactant production ability by hemolytic activity and drop collapsing test. All isolated four strain $APCCS_{1a,}$ $APCCS_{1b,}$ $APCCS_{2b\ and}$ $APCCS_{5a}$ give positive in hemolytic activity among them first and second are beta hemolytic (complete) and third and fourth are alpha hemolytic (incomplete). Finally drop collapsing test was done as by Youssef et al., 2004 of isolated culture and flat drops were scored as positive '+' score biosurfactant production.

The strains are characterized using different morphological and biochemical process using Bergey's

manual as reference. The isolated strains werebelonging to Pseudomonas (APCCS1a and APCCS5a), bacillus (APCCS1b) and Lactobacillus (APCCS2b) groups. The surfactant from each of these four strains were extracted by culturing in liquid medium (Mcknee medium) using convention technique and further characterized by thin layer chromatography using Ninhydrin and anthrone reagent as developing solution. This shows the production of lipopeptide (for $APCCS_{1b}$) and glycolipid (for$APCCS_{1a}$, $APCCS_{3b}$ and $APCCS_{5a}$) biosurfactants in the organisms. The emulsification activities of each strain are not same for all four isolated strain. The emulsification activity was measured according to the method of Cooper and Goldenberg (1987) and the result show that $APCCS_{1a}$ and $APCCS_{5a}$ has highest emulsifying activity whereas $APCCS_{1b}$ lowest. The isolated strain are tested in all experiment in triplicate manner and best represented here indicate that the natural environment may be act as good source for alternative of toxic chemical surfactant.

5. Conclusion:

Uses of biosurfactants are increasingly in almost every sectors of the modern industry as an alternative to chemical surfactants. With increasing public awareness in the environment, biosurfactant would most likely replace the usage of chemical surfactants in the near future. As biosurfactants are derived from natural sources, each of these types is an attractive alternative to synthetic compounds

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